Accepted Manuscript

Title: Dynamics of *Haemophilus parasuis* genotypes in a farm recovered from an outbreak of Glässer’s Disease

Authors: A. Olvera, M. Cerdà-Cuéllar, M. Nofrarías, E. Revilla, J. Segalés, V. Aragon

PII: S0378-1135(07)00137-X
DOI: doi:10.1016/j.vetmic.2007.03.004
Reference: VETMIC 3614

To appear in: **VETMIC**

Received date: 7-2-2007
Revised date: 1-3-2007
Accepted date: 8-3-2007

Please cite this article as: Olvera, A., Cerdà-Cuéllar, M., Nofrarías, M., Revilla, E., Segalés, J., Aragon, V., Dynamics of *Haemophilus parasuis* genotypes in a farm recovered from an outbreak of Glässer’s Disease, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.03.004

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Dynamics of *Haemophilus parasuis* genotypes in a farm recovered from an outbreak of Glässer’s Disease

**Running Title:** Dynamics of *H. parasuis* strains genotypes

A. Olvera¹, M. Cerdà-Cuéllar¹, M. Nofrarias¹, E. Revilla², J. Segalés¹,³ and V. Aragon¹

² Albet, S.A. Perot Rocaguinarda 18. 08500-Vic. Barcelona. Spain

*Corresponding author:* Virginia Aragon

Centre de Recerca en Sanitat Animal (CReSA). Campus de Bellaterra-Universitat Autònoma de Barcelona. 08193-Bellaterra, Barcelona (Spain).

Phone: +34 93 581 4494
Fax: +34 93 581 4490

e-mail: virginia.aragon@cresa.uab.es
Abstract

*Haemophilus parasuis* is a colonizer of the upper respiratory tract of pigs, although it is better known as the etiological agent of Glässer’s disease. Interestingly, several strains can be isolated from a single farm, as determined by both genotyping and serotyping. However, it is not known how an outbreak and the subsequent treatment affect the population of *H. parasuis* strains. In this study, a farm was studied during an outbreak of Glässer’s disease and one year after antimicrobial treatment and elimination of clinical signs. Bacterial isolation was attempted from nasal swabs and lesions. After isolation, antimicrobial susceptibility, serotype and genotype were determined. Two different genotyping techniques, enterobacterial repetitive intergenic consensus (ERIC)-PCR and multilocus sequence typing (MLST) were used. The *H. parasuis* strain that was isolated from lesions during the disease outbreak clustered with other virulent strains by both MLST and serotyping analysis. Nasal isolates were included in the corresponding nasal cluster by MLST, but they presented high variability by serotyping. These nasal isolates included serotypes previously classified as virulent and non-virulent. Finally, we found that during the antimicrobial treatment the diversity of strains isolated in the farm was affected and just one strain, which was resistant to the treatment, was detected. One year after the treatment strain diversity was back to normal (3 strains).

Keywords: *Haemophilus parasuis*, Glässer’s disease, field strains, genotyping
Introduction

_Haemophilus parasuis_ is the etiological agent of Glässer’s disease in pigs, which is characterized by serofibrinous to fibrinopurulent polyserositis, arthritis and meningitis (Nicolet, 1968). _H. parasuis_ is also involved in other clinico-pathological outcomes, such as pneumonia and sudden death, and causes high morbidity and mortality in naïve swine populations (Miniats et al., 1986). On the other hand, _H. parasuis_ colonizes the upper respiratory tract of pigs at a very young age and, therefore, it can be isolated from the nose of healthy pigs (Harris et al., 1969; Møller and Kilian, 1990; Oliveira and Pijoan, 2004).

_H. parasuis_ strains differ in several features, including pathogenic potential (Rapp-Gabrielson et al., 2006). Classically, strains of _H. parasuis_ have been classified by serotyping and, although some serotypes seem to be more pathogenic than others (Rosner et al., 1991; Rapp-Gabrielson et al., 1992; Nielsen, 1993; Amano et al., 1994, Amano et al., 1996), the correlation between serotype and virulence is not complete. More importantly, a significant percentage of isolates, between 15 and 41%, are non-typeable by serotyping (Oliveira and Pijoan, 2004). In order to circumvent this problem, fingerprinting and sequencing methods have been developed to assess the heterogeneity of _H. parasuis_ isolates by genotyping (Olvera et al., 2006c). The application of enterobacterial repetitive intergenic consensus (ERIC)-PCR to _H. parasuis_ is especially useful in local epidemiology (i.e. to assess the strains circulating within a farm) (Ruiz et al., 2001; Oliveira et al., 2003), but the results of this technique are hardly portable and difficult to compare among laboratories. Thus, in order to perform global epidemiology studies, other genotyping methods have been developed (Olvera et al., 2006a; Olvera et al., 2006b). Sequence typing, especially multilocus sequence typing (MLST), reported
clusters of *H. parasuis* strains associated with disease production or, on the contrary, with nasal microbiota (Olvera et al., 2006b).

Using different genotyping methods, it has been shown that several strains can be isolated from a single conventional farm and that strains associated with respiratory pathology differ from systemic ones (Smart et al., 1988; Smart et al., 1993; Ruiz et al., 2001; Oliveira et al., 2003; Olvera et al., 2006a; Olvera et al., 2006b). However, it is not known how an episode of Glässer’s disease and the subsequent treatment with antimicrobials affect the population of *H. parasuis* strains in a farm. Thus, the goal of this work was to study *H. parasuis* strain dynamics in a farm during an outbreak of Glässer’s disease and a year later, using serotyping and two different genotyping techniques.

**Material and Methods**

**Case farm**

In October 2004, an outbreak of Glässer’s disease was diagnosed in a 300-sow, farrow-to-finish farm located in Northeastern Spain. Clinical signs, including dyspnoea, diarrhoea and sudden death, were observed at about 5 weeks of age (weaning was performed at ~24 days of age). On-farm necropsies showed fibrinous polyserositis, and lungs from four affected pigs were submitted for bacterial isolation to the diagnostic service of the Department of Infectious Diseases of the Veterinary School at the Universitat Autònoma de Barcelona (UAB) in Spain. *H. parasuis* was isolated from one of these lungs (isolate 228/04), and antimicrobial sensitivity determination was performed. The veterinarian decided to start a treatment with amoxicillin (feed treatment, 400 ppm).
After one month of amoxicillin treatment (November 2004), a similar clinical-pathological problem (with fibrinous polyserositis) was still present in the farm, but affecting the growing units (9-11 week-old pigs). Therefore, the antimicrobial treatment was changed and a combination of phenoximetilpenicilin and tylosin was applied (feed treatment, 200 ppm and 100 ppm, respectively). At that time, two 9 week-old pigs (CD12 and CD13) were found dead and, at necropsy, sero-fibrinous fluids of thoracic and abdominal cavities were taken for bacterial isolation. Besides, eleven nasal swabs (CD1 to CD11), sampling both nostrils, were taken from 6 to 11 week-old pigs from the units with clinical disease. The established antimicrobial treatment apparently worked and no further cases compatible with Glässer’s disease were reported.

One year after the initial outbreak (October 2005), the farm remained free of Glässer’s disease based on clinical and pathological observations. At that time, ten nasal swabs from 6 to 8 week-old pigs (CN1 to CN10) were taken for *H. parasuis* isolation and the subsequent typing analysis.

**H. parasuis** isolation and identification

Collected swabs were placed in Amies medium and transported under refrigeration to the laboratory, where they were plated on chocolate agar to isolate colonies. After 2 to 3 days at 37°C with 5% CO₂, all *H. parasuis*-like colonies were selected and subcultured for further analysis. A species-specific PCR (Oliveira et al., 2001) and classical biochemical tests (Møller and Kilian, 1990) to identify *H. parasuis* were performed, although final identification was accomplished by 16S rRNA gene sequencing (GenBank accession numbers DQ228977, DQ228980, DQ228981, DQ228983 to DQ228985, EF396294, EF396297 to EF396303). Bacterial suspensions in sterile phosphate buffer saline (PBS) were used to extract genomic DNA from each isolate.
The extraction was performed with the Instagene DNA purification Matrix 2 (Bio-Rad Laboratories Inc., Spain) following manufacturer instructions. A fragment of the 16S rRNA gene of about 1400 bp was amplified and sequenced as reported previously (Olvera et al., 2006a). The resulting sequences were used in database searches using the BLAST algorithm (Altschul et al., 1997). Both the NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the Ribosomal Database Project (http://rdp.cme.msu.edu) databases were searched. A threshold of 99% sequence identity was used for species identification (Janda and Abbott, 2002).

Antimicrobial susceptibility

Antimicrobial susceptibility tests were performed using the Kirby-Bauer method according to CLSI guideline M31-A2, with some modifications. Briefly, a 0.5 MacFarland suspension of each isolate was made from a 24 h culture on chocolate agar plates. Each suspension was spread on chocolate agar plates using a swab and discs of seven different antimicrobials (Neo-sensitabs™, Rosco Diagnostica, Denmark) were placed on the plates, which were then incubated for 24 h at 37°C with 5% CO₂. The antimicrobials tested were amoxicillin (30 µg), ceftifour (30 µg), enrofloxacin (10 µg), doxycycline (80 µg), trimethoprim-sulphomethoxazole (5.2 + 240 µg) and tylosin (150 µg).

Serotyping

Serotype determination was performed by indirect hemagglutination at the Department of Sanidad Animal of the Veterinary School at the University of Leon (Spain) following a previously published protocol (del Rio et al., 2003). Soluble antigen was obtained
after boiling a bacterial suspension and subsequent centrifugation to eliminate insoluble debris.

Multilocus sequence typing (MLST)

Fragments of the *rpoB, 6pgd, mdh, infB, frdB, g3pd* and *atpD* genes of *H. parasuis* were amplified as previously published (Olvera et al., 2006a). Amplicons were sequenced using the same PCR primers and BigDye terminator v.3.1 kit, using an ABI 3100 DNA sequencer (Applied Biosystems, USA). Sequences were deposited at the GenBank database (accession numbers DQ781634 to DQ781637, DQ781660, DQ781661, DQ781663, DQ781666, DQ781673, DQ781503 to DQ781506, DQ781529, DQ781530, DQ781532, DQ781535, DQ781542, DQ782289 to DQ782292, DQ782315, DQ782316, DQ782318, DQ782321, DQ782328, DQ782158, DQ782159, DQ782184, DQ782185, DQ782187, DQ782190, DQ782197, DQ782027 to DQ782030, DQ782053, DQ782054, DQ782056, DQ782059, DQ782066, DQ782896 to DQ782899, DQ78292, DQ78293, DQ782925, DQ782928, DQ782935, DQ782765 to DQ782768, DQ782791, DQ782792, DQ782794, DQ782797, DQ782804, EF396288 to EF396293, EF396308 to EF396322).

Sequence editing and allele assignment was performed with Fingerprinting II v3.0 software (BioRad, USA). MLST analysis was carried out using START2 (Jolley et al., 2001) by the neighbour-joining algorithm (NJ) based on allelic profiles.

Enterobacterial repetitive intergenic consensus (ERIC)-PCR

Purified DNA was quantified by spectrometry and 100 ng were used as template in ERIC-PCR. The technique followed a previously published protocol (Oliveira et al., 2003), including an extra final extension step of 20 min (Olvera et al., 2006a). Images of the gels were captured with a ChemiGenius transiluminator (SynGene Ltd. Frederick,
MD, USA.) and stored as TIFF files for further analysis. Bands from 4,000 to 100 bp were used in the analysis. ERIC-PCR fingerprints analysis, sequence editing and analysis were performed using the Fingerprinting II v3.0 software. Finally, ERIC-PCR band patterns were normalized and a Pearson correlation similarity matrix calculated. Cluster analysis of ERIC-PCR fingerprints were performed by UPGMA (Ooyen, 2001).

Results and discussion

Several studies have examined the variability of *H. parasuis* strains circulating in a farm by genotyping and serotyping, but none of them has followed up the strain population over time. Moreover, there are several studies on the antimicrobial susceptibility of *H. parasuis*, but, as far as we know, this is the only work that studies the effect of antimicrobial treatment on *H. parasuis* strain variability within the same farm.

When disease outbreak was observed (October 2004), *H. parasuis* was isolated from one of the 4 lungs from sacrificed animals with fibrinous polyserositis (isolate 228/04). Antimicrobial susceptibility tests showed that isolate 228/04 was sensitive to all the antimicrobials tested (Table 1). Amoxicillin treatment was implemented but, after one month (November 2004), the clinical problem moved to the growing units. Two animals (CD12 and CD13) were found dead and after necropsia, *H. parasuis* DNA was detected by PCR in abdominal and thoracic fluids. However, *H. parasuis* could not be isolated from the lesions of these pigs. These results can be explained by the fastidious growth requirements of this microorganism and its difficult isolation from already dead animals. At that time, a total of 25 *H. parasuis* isolates were obtained from eleven nasal swabs (isolates CD1 to CD11). Genotyping of these nasal isolates showed that they had the same ERIC-PCR profile (Fig. 1), no differences in 16S rRNA gene sequence (data
not shown) and the same MLST sequence type (ST) (Fig. 1), indicating that they were a single strain. The antimicrobial susceptibility tests of several \( H. parasuis \) CD isolates demonstrated that those isolates were highly resistant to amoxicillin and sensitive to the rest of antimicrobials tested (Table 1). Antimicrobial treatment had a clear effect on the population of \( H. parasuis \) in this farm. Clearly, the use of amoxicillin to control the outbreak selected one clone (\( H. parasuis \) CD) that was resistant to this antimicrobial and induced a homogenization of the strain population. Apparently, strain 228/04 was eliminated by the treatment, since it was not isolated anymore.

Besides antimicrobial susceptibility, \( H. parasuis \) CD isolates and strain 228/04 showed clear genotypic differences (Fig. 1), indicating that the \( H. parasuis \) CD strain and 228/04 were indeed different strains. These results may indicate that two strains, 228/04 and \( H. parasuis \) CD, were initially associated with clinical problems and the antimicrobial treatment eliminated just one or, alternatively, that the elimination of the first one (228/04) was an opportunity for the second one (CD) to proliferate and cause a second round of disease. On the other hand, it can not be ruled out that the first one was never the cause of the disease. Actually, 228/04 was isolated from lung and a clear link with the observed systemic disease is not evident, since a lung isolate may not necessarily be the cause of a septicaemia. This is a major problem with all \( H. parasuis \) lung isolates from Glässer’s disease cases, together with the difficulties to discard them as postmortem invasion from the upper respiratory tract. The global comparison of strains by MLST (Fig 2) supported that 228/04 was indeed the cause of the disease since it was included in a cluster associated with disease production and formed mainly by systemic isolates (Olvera et al., 2006b). Moreover, this strain belonged to serotype 5, which has been repeatedly demonstrated highly virulent in experimental infections (Kielstein and Rapp-Gabrielson, 1992; Nielsen, 1993; Amano et al., 1994). On the other
Hand, *H. parasuis* CD isolates were included in a MLST cluster with strains of nasal origin (Fig. 2), indicating that they were probably a non-virulent strain not implicated in the clinical outbreak. Curiously, when the serotype was determined for 3 of those isolates, they belonged to serotypes 10, 14 and 15; although they had the same genotype (Fig 1). Taking into account the rate of evolution of ERIC-PCR and MLST patterns this could mean that serotype in *H. parasuis* is an unstable phenotypic characteristic that can change relatively fast, at least faster than genomic rearrangements or mutations. Alternatively, *H. parasuis* serotyping needs further optimization to avoid this variability, which could be due to a certain degree of crossreactivity between serotypes.

Although no other *H. parasuis* strain was isolated in the affected growing units during the clinical problem, association of *H. parasuis* CD strain with disease onset is, obviously, difficult to establish because it was isolated from nasal swabs. Unfortunately, no *H. parasuis* could be isolated from the lesions of pigs CD12 and CD13, although the bacterium was detected by the *H. parasuis*-specific PCR. Unexpectedly, ascites from pig CD12 yielded a *Moraxella spp* isolate (CD12CA4), as identified by 16S rRNA gene sequencing (best Blastn hit with *Moraxella cuniculi* [Accession number AF005188] with 97% of sequence identity). This result indicates the possibility that the clinical signs observed in the growing units were due to this opportunistic pathogen. Nevertheless, the association of *Moraxella spp* with disease onset in pigs is difficult to establish. Some authors have reported the isolation of *Moraxella*-like microorganisms from polyserositis lesions of pigs, but the pathogenicity of these isolates could not be demonstrated in experimental infections (Larsen et al., 1973; Nielsen and Danielsen, 1975).
Finally, the treatment was changed to phenoximethylpenicillin and tylosin and the problem was controlled (all the isolates of this study were sensitive, at least, to tylosin) and the *H. parasuis* CD strain was eliminated. After one year, in which the farm remained free of Glässer’s disease, ten 6 to 8–week-old pigs were sampled and a total of 23 isolates of *H. parasuis* were obtained (isolates CN followed by numbers). Three different strains were identified among these CN isolates (represented by CN9-2, CN10-1 and the group of CN8-1; Fig 1) by both genotyping methods (ERIC-PCR and MLST). Thus, the variability of strains was back to normal (usually, 3-5 strains/farm) (Smart et al., 1988; Smart et al., 1989; Smart et al., 1993; Rafiee et al., 2000). When the MLST analysis was done with 205 strains from different origins, all CN isolates were included in the cluster of strains associated with nasal isolation (Fig. 2) (Olvera et al., 2006b). Serotype was determined for a representative of each genotype and serotypes 9 and 15 were reported (Fig. 1). These isolates showed different susceptibilities to amoxicillin (Table 1), but all of them were sensitive to the rest of antimicrobials tested. CN10-1 was resistant to amoxicillin (Table 1) and showed the closest genotype to the CD isolates, with just one different allele (*infB* 16) in the MLST allelic profile. Allele *infB* 16 was also present in the isolates from the group of CN8-1. The CN10-1 *infB* allele (*infB* 16) differed in two nucleotides with the corresponding allele in the CD strains (*inf 8*) and was identical to the CN8-1 *infB* allele. The detection of homologous recombination in *H. parasuis* in a previous work (Olvera et al., 2006b) gives more support to the occurrence of one homologous recombination event between the CD and the CN8-1 isolates, instead of two independent point mutation events. Indeed, it is very likely that CN10-1 is a descendent of the *H. parasuis* CD strain that has exchanged the *infB* 16 allele with the CN8-1 isolates. The origin of the remaining CN strains
(represented by isolates CN2-1 and CN9-2) is not fully clear given that they were very different from those detected during the clinical outbreak. It is possible that those CN strains were in the farm prior to the outbreak. Thus, the use of antimicrobial treatment induced the elimination of them in the units were the treatment was applied, but they were reintroduced by the sows. Therefore, they might represent the situation of the farm before the clinical outbreak. However, it cannot be ruled out that those CN strains were introduced by replacement breeding stock.

The genotyping methods used in this study, ERIC-PCR and MLST, were useful in the assessment of the number of clones present in a farm, and yielded similar results. For the identification of the different clones in a farm, ERIC-PCR is faster and less expensive than MLST. However, MLST produces additional information, since it also allows the comparison of the isolates in a particular farm with other epidemiologically-unrelated isolates. The comparison of the isolates obtained in the case farm with strains from different clinical and geographical origins (Olvera et al., 2006b) was helpful to elucidate the putative virulence of the strains.

In summary, this study indicates that the antimicrobial treatment allowed the emergence of a resistant strain, which increased in frequency and resulted in the only \textit{H. parasuis} strain isolated, but the strain diversity could return to normality in less than a year after the episode of Glässer's disease.

Acknowledgements

We thank Núria Galofré for technical support and the Diagnostic Service of the Veterinary School at University Autònoma of Barcelona for providing us with isolate 228/04. This work was funded by grant AGL2004-07349 from the Ministerio de
Educación y Ciencia of Spain. Fellowship support for A. O. from CReSA is also acknowledged.

References


**Figure legends**

**Figure 1.** Genotyping and serotyping of *H. parasuis* isolates. ERIC-PCR fingerprints were analyzed by UPGMA and MLST allelic profiles by neighbour-joining. MLST allelic profiles correspond to the alleles of *rpoB, 6pgd, mdh, infB, frdR, g3pd* and *atpD*. Strain 228/04 was isolated from lung lesions during a Glässer’s disease outbreak, CD isolates were obtained from nasal swabs after 1 month of amoxicillin treatment and CN isolates were obtained from nasal swabs 1 year later. Isolates corresponding to a single strain are included in the same box. *ND, not determined.*

**Figure 2.** Neighbour-joining dendogram constructed using the allelic profiles of loci *rpoB, 6pgd, mdh, infB, frdR, g3pd* and *atpD* of 205 *H. parasuis* isolates. Isolates from the case farm are indicated in the dendogram. Additionally, non-virulent reference strains D74, SW114, C5 and virulent reference strains Nagasaki, H367, 84-22113, 84-15995 are also indicated.
Table 1. Antibiotic susceptibility of the different isolates studied.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Bacteria</th>
<th>AM OXY</th>
<th>TR+SU</th>
<th>ENROF</th>
<th>DOXYC</th>
<th>CFTIO</th>
<th>TYLOS</th>
<th>Isolation date</th>
</tr>
</thead>
<tbody>
<tr>
<td>228/04</td>
<td><em>H. parasuis</em></td>
<td>S (52)</td>
<td>S (39)</td>
<td>S (41)</td>
<td>S (39)</td>
<td>S (57)</td>
<td>S (33)</td>
<td>10/2004</td>
</tr>
<tr>
<td>CN10-1</td>
<td><em>H. parasuis</em></td>
<td>R (0)</td>
<td>S (28)</td>
<td>S (34)</td>
<td>S (32)</td>
<td>S (38)</td>
<td>S (26)</td>
<td>10/2005</td>
</tr>
</tbody>
</table>

1 AM OXY: amoxicillin (30 µg); DOXYC: doxycycline (80 µg); ENROF: enrofloxacin (10 µg); TR+SU: trimethoprim-sulphomethoxazole (5.2 + 240 µg); CFTIO: ceftifour (30 µg); TYLOS: tylosin (150 µg).

2 Inhibition diameter in mm is indicated in parenthesis.
No natural text can be extracted from this page.